MICHIGAN MEDICINE

Translational Tissue Modeling Laboratory

Generation of LWRN Conditioned Media

Protocol based on Miyoshi et al, 2013 PMC3969856

Protocol authors: Angeline Wu, Yuhwai Tsai, Stacy Finkbeiner, Michael Dame, Jason Spence.
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Description:
This protocol describes how to generate media containing mouse Wnt-3A, R-spondin 3, and Noggin used for generating and maintaining mouse and human enteroid/colonoid cultures.

Equipment & Materials:

- Tissue Culture Hood
- L-WRN Cells (ATCC CRL-3276)
    - Growth Media: Advanced DMEM/F12 (Gibco, 12634-028) + 20% FBS (Gibco, 26140-079; ≤10EU/mL) + 1% GlutaMAX (Gibco, 35050-061) + 1% Pen/Strep (final 100 U/mL; Gibco, 15140-122)
    - Selection: Geneticin/G418 & Hygromycin (both 0.5 mg/mL)
- 15 cm Petri Dishes
- Greiner Bio-One CELLdisc™ Multilayer Cell Culture Surface (Fisher 07001057) for large-scale productions
- 500 mL Vacuum Filtration Units (ThermoFisher 569-002 Rapid Flow 0.2µM PES membrane 90mm filter)
- 250-500 mL Sterile PETG Media Bottles (ThermoFisher FBMB250)
- 50 mL Conicals
- 1.5 mL Centrifuge Tubes

Methods:

1. Thaw LWRN cells in 30mL Growth Media and culture the cells in one 15cm Petri Dish.
2. The next day, depending on cell recovery, change media with dual selection and grow to about 80-90% confluency (usually ~ five days) without media change.
3. Split cells into 10 new plates without antibiotic selection.
4. Add 25 mL of Growth Media per plate.
5. Once cells are about 80% confluent (usually ~2 days), collect and replace media on each plate **every day** for 10-12 consecutive days.
6. Save 500 µL of media into a centrifuge tube from each day for testing activity using the TOPflash Wnt Reporter and mycoplasma testing. Assay and store at 4°C.
7. Spin at 500xg for 10 minutes, or, allow debris to sediment overnight at 4°C. The following morning, filter sterilize and aliquot into 25 or 50 mL volumes and store aliquots at -80°C. (e.g., filtering, aliquoting, and freezing media from day 1 on Day 2).

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LWRN QC

1. Limit freeze-thaws to 2 times. Once thawed, the LWRN is functional for 14 days when stored at 4C (VanDussen et al, 2019 PMC6579736)
2. L-WRN conditioned media contains approximately 45 ng/mL Wnt-3A, 25ng/mL R-spondin 3, and 25 ng/mL noggin (Speer, JE… Nancy L. Allbritton. Evaluation of human primary intestinal monolayers for drug metabolizing capabilities. J Biol Eng 13, 82 (2019) doi:10.1186/s13036-019-0212-1)
3. The TTML also performs a Wnt assay and uses this as a guide. The below 7 day functional test on each collection day ultimately determines usage.

Functional QC

1. We test one of our most sensitive colonoid lines, which is Colon-87.
2. Each LWRN collection day is used to set up a well of colonoids (24-well plate; four 10uL drops each well). Medium is also added to the matrigel as described in our methods.
3. Two positive controls: IntestiCult-human (StemCell Technologies; 06010) and a good performing mix from the previous batch.
4. Negative control: usually day-1 or the late days, 10-12, will serve as an internal low performer.
5. Medium is refreshed every day.
6. At the end of the 7 days we document by imaging and also blindly count dead/differentiated structures for each day.
7. Based on results, we combine individual high and low proliferation days and retest. Ideally, we test and refreeze mixes from 3-4 days in working volumes of 50 mLs.

    a. See this QC example. If the medium produces a majority of structures that are grey/black, we will likely not use it at all (Day 1/2 example), unless we have human fetal or mouse colonoids in culture which requires less Wnt.

    b. If some shiny structures are evident (Day 9/10 or 11/12 example) then this suggests new growth and we will attempt to salvage that day by combining it with medium that had very minimal death. In this example we may combine Day 5/6, 7/8, and 11/12. The first time we make this particular combination, with each individual batch, we will test alongside a working combination. We define a working combination as one that produces approximately 90% clear shiny cystic structures in Colon-87. Most of the time we hit the mark, but occasionally we are surprised and have to adjust, that is, we have to add another high-performing day to the mix.

    c. So the goal is not maximal activity for colonoid maintenance. For certain applications we do indeed push towards maximal activity, such as preparation for 2D monolayers.